pENTR Directional TOPO® Cloning Kits

Five-minute, directional TOPO® Cloning of blunt-end PCR products into an entry vector for the Gateway™ System

Catalog nos. K2400-20, K2400-480, K2400-500, K2420-20, K2420-480, K2420-500

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Table of Contents

TOPO® Cloning Procedure for Experienced Users

Introduction

This quick reference sheet is provided for experienced users of the TOPO® Cloning procedure. If you are performing the TOPO® Cloning procedure for the first time, we recommend that you follow the detailed protocols provided in the manual.

Step	Action
Design PCR Primers	• Include the 4 base pair sequences (CACC) necessary for directional cloning on the 5′ end of the forward primer. • Design the primers such that your gene of interest will be optimally expressed and fused in frame with any epitope tags, if desired (after recombination with the Gateway™ destination vector).
Amplify Your Gene of Interest	1. Use a thermostable, proofreading DNA polymerase and the PCR primers above to produce your blunt-end PCR product. 2. Use agarose gel electrophoresis to check the integrity of your PCR product.
Perform the TOPO® Cloning Reaction	1. Set up the following TOPO® Cloning reaction. **Note:** If you plan to transform electrocompetent *E. coli*, use Dilute Salt Solution in the TOPO® Cloning reaction. Fresh PCR product — 0.5 to 4 µl Salt Solution — 1 µl Sterile water — add to a final volume of 5 µl TOPO® vector — 1 µl Total volume — 6 µl 2. Mix gently and incubate for 5 minutes at room temperature. 3. Place on ice and proceed to transform One Shot® TOP10 chemically competent *E. coli*, below.
Transform TOP10 Chemically Competent *E. coli*	1. Add 2 µl of the TOPO® Cloning reaction into a vial of One Shot® TOP10 chemically competent *E. coli* and mix gently. 2. Incubate on ice for 5 to 30 minutes. 3. Heat-shock the cells for 30 seconds at 42°C without shaking. Immediately transfer the tube to ice. 4. Add 250 µl of room temperature SOC medium. 5. Incubate at 37°C for 1 hour with shaking. 6. Spread 50-200 µl of bacterial culture on a prewarmed selective plate and incubate overnight at 37°C.

Control Reaction

We recommend using the Control PCR Template and the Control PCR Primers included with the 20 reaction kits to perform the control reaction. See the protocol on pages 22-24 for instructions.

HTP TOPO® Cloning Procedure for Experienced Users

Introduction

This quick reference sheet is provided for experienced users of the HTP TOPO® Cloning procedure. If you are performing the HTP TOPO® Cloning procedure for the first time, follow the detailed protocols provided in the manual.

<table>
<tr><th>Step</th><th colspan="2">Action</th></tr>
<tr><td>Design PCR Primers</td><td colspan="2">• Include the 4 base pair sequences (CACC) necessary for directional cloning on the 5′ end of the forward primer.
• Design the primers such that your gene of interest will be optimally expressed and fused in frame with any epitope tags, if desired (after recombination with the Gateway™ destination vector).</td></tr>
<tr><td>Amplify Your Gene of Interest</td><td colspan="2">1. Use a thermostable, proofreading DNA polymerase and the PCR primers above to produce your blunt-end PCR product.
2. Use agarose gel electrophoresis to check the integrity of your PCR product.</td></tr>
<tr><td>Perform the TOPO® Cloning Reaction</td><td colspan="2">1. Set up the following TOPO® Cloning reaction in each well of a 96-well plate. Note: If you plan to transform electrocompetent E. coli, include Dilute Salt Solution in the TOPO® Cloning reaction.
Fresh PCR product 1 µl
Salt Solution 1 µl
Sterile water 3 µl
TOPO® vector 1 µl
Total volume 6 µl
2. Incubate for 5 minutes at room temperature.
3. Place the 96-well plate on a cooling block for 5 minutes.</td></tr>
<tr><td>Transform TOP10 Chemically Competent E. coli</td><td>Bulk Cells
1. Pour TOP10 E. coli into a sterile trough. Aliquot 45 µl of cells into each well. Gently pipet up and down to mix.
2. Cover plate and incubate on a chilled block for 20 minutes.
3. Heat-shock the cells at 42°C for 30 seconds.
4. Transfer plate to cooling block and incubate for 1 minute.
5. Add 150 µl/well of SOC.
6. Incubate at 37°C for 1 hour with shaking.
7. Plate 50 µl from each well onto selective plates. Incubate overnight at 37°C.</td><td>Cells in 96-well Plates
1. Place 96-well plate on a cooling block and thaw TOP10 cells for 30 seconds.
2. Add 2 µl of each TOPO® Cloning reaction to each well of the 96-well plate.
3. Incubate on a chilled block for 20 minutes.
4. Heat-shock the cells at 42°C for 30 seconds.
5. Transfer plate to cooling block and incubate for 1 minute.
6. Add 90 µl/well of SOC.
7. Incubate at 37°C for 1 hour with shaking.
8. Plate 100 µl from each well onto selective plates. Incubate overnight at 37°C.</td></tr>
</table>

Kit Contents and Storage

Types of Kits

This manual is supplied with the following kits.

Kit	Reactions	Catalog no.
pENTR Directional TOPO® Cloning Kit	20	K2400-20
	480	K2400-480
	500	K2400-500
pENTR/SD Directional TOPO® Cloning Kit	20	K2420-20
	480	K2420-480
	500	K2420-500

Shipping/Storage

The pENTR and pENTR/SD Directional TOPO® Cloning Kits are shipped on dry ice. Each kit contains two boxes as described below. Upon receipt, store the boxes as detailed below.

Box	Item	Storage
1	pENTR TOPO® Reagents	-20°C
2	TOP10 Chemically Competent *E. coli*	-80°C

continued on next page

Kit Contents and Storage, continued

pENTR TOPO® Reagents

pENTR TOPO® reagents (Box 1) are listed below. **Note that the user must supply a thermostable, proofreading polymerase and the appropriate PCR buffer.**

Store Box 1 at -20°C.

Item	Concentration	Amount/ 20 Rxn Kit	Amount/ 480 or 500 Rxn Kit
pENTR TOPO® vector, TOPO®-adapted (pENTR/D-TOPO® **or** pENTR/SD/D-TOPO®)	5-10 ng/µl linearized plasmid DNA in: 50% glycerol 50 mM Tris-HCl, pH 7.4 (at 25°C) 1 mM EDTA 2 mM DTT 0.1% Triton X-100 100 µg/ml BSA 30 µM bromophenol blue	20 µl	500 µl
dNTP Mix	12.5 mM dATP 12.5 mM dCTP 12.5 mM dGTP 12.5 mM dTTP in water, pH 8	10 µl	300 µl
Salt Solution	1.2 M NaCl 0.06 M $MgCl_2$	50 µl	500 µl
Sterile Water	---	1 ml	3 x 1 ml
M13 Forward (-20) Sequencing Primer	0.1 µg/µl in TE Buffer, pH 8	20 µl	2 x 20 µl
M13 Reverse Sequencing Primer	0.1 µg/µl in TE Buffer, pH 8	20 µl	2 x 20 µl
Control PCR Primers	0.1 µg/µl **each** in TE Buffer, pH 8	10 µl	Not included
Control PCR Template	0.1 µg/µl in TE Buffer, pH 8	10 µl	Not included

Sequences of the Primers

The table below provides the sequences of M13 Forward and M13 Reverse sequencing primers.

Primer	Sequence	pMoles Supplied
M13 Forward (-20)	5′-GTAAAACGACGGCCAG-3′	407
M13 Reverse	5′-CAGGAAACAGCTATGAC-3′	385

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Kit Contents and Storage, continued

TOP10 Chemically Competent Cell Format

TOP10 Chemically Competent *E. coli* are supplied in a variety of formats depending on the catalog number (see table below).

Catalog no.	Competent Cell Format	Reactions
K2400-20 and K2420-20	• 20 x 50 µl in One Shot® format • 1 x 50 µl in One Shot® format (for control)	20
K2400-480 and K2420-480	• Five 96-well plates in MultiShot™ format (15 µl/well in stripwells) • 1 plate with 12 wells of cells only (for control)	480
K2400-500 and K2420-500	• 5 x 5 ml in Bulk TOP10 format • 1 x 50 µl (for control)	500

TOP10 Chemically Competent Cell Reagents

The table below lists the items included in the TOP10 Chemically Competent *E. coli* kit (Box 2). Transformation efficiency is 1 x 10^9 cfu/µg DNA for cells supplied in the 20 reaction kit and 1 x 10^8 cfu/µg DNA for cells supplied in the 480 and 500 reaction kits. **Store Box 2 at -80°C.**

Item	Composition	Amount/ 20 Rxn Kit	Amount/ 480 Rxn Kit	Amount/ 500 Rxn Kit
SOC Medium (may be stored at room temperature or +4°C)	2% Tryptone 0.5% Yeast Extract 10 mM NaCl 2.5 mM KCl 10 mM $MgCl_2$ 10 mM $MgSO_4$ 20 mM glucose	6 ml	5 x 15 ml	
TOP10 cells	--	20 x 50 µl	5 x 96-well plates 15 µl/well in strip-wells	5 x 5 ml
Control TOP10 cells		1 x 50 µl	Test plate with 12 wells of cells (1 row)	1 x 50 µl
pUC19 Control DNA	10 pg/µl in 5 mM Tris-HCl, 0.5 mM EDTA, pH 8	50 µl		

Genotype of TOP10

F⁻ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *deo*R *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G

Accessory Products

Introduction

The products listed in this section may be used with the pENTR Directional TOPO® Cloning Kits. For more information, refer to our Web site (www.invitrogen.com) or call Technical Service (see page 32).

Additional Products

Many of the reagents supplied in the pENTR Directional TOPO® Cloning Kits and other reagents suitable for use with the kits are available separately from Invitrogen. Ordering information for these reagents is provided below.

Item	Quantity	Catalog no.
One Shot® TOP10 **Chemically Competent** *E. coli*	10 reactions	C4040-10
	20 reactions	C4040-03
One Shot® TOP10 **Electrocompetent** *E. coli*	10 reactions	C4040-50
MultiShot™ TOP10 Chemically Competent *E. coli*	5 plates	C400-05
M13 Forward (-20) Primer	2 µg (407 pmoles)	N520-02
M13 Reverse Primer	2 µg (385 pmoles)	N530-02
Platinum® *Pfx* DNA Polymerase	100 units	11708-013
Kanamycin Sulfate	1 g	11815-016
LB Broth	500 ml	10855-021
LB Agar	500 g	22700-025
S.N.A.P.™ MidiPrep Kit	20 reactions	K1910-01
Gateway™ LR Clonase™ Enzyme Mix	20 reactions	11791-019

Introduction

Overview

Introduction

The pENTR and pENTR/SD Directional TOPO® Cloning Kits utilize a highly efficient, 5-minute cloning strategy ("TOPO® Cloning") to directionally clone a blunt-end PCR product into a vector for entry into the Gateway™ System available from Invitrogen. Blunt-end PCR products clone directionally at greater than 90% efficiency, with no ligase, post-PCR procedures, or restriction enzymes required.

A choice of pENTR Directional TOPO® vectors is available for optimal expression of your PCR product after recombination with the Gateway™ destination vector of interest (see Table below).

Vector	Benefit
pENTR/D-TOPO®	For efficient expression of your gene of interest after recombination with a Gateway™ destination vector
pENTR/SD/D-TOPO®	Contains a T7 gene 10 translational enhancer and a ribosome binding site (RBS) for optimal expression of native protein after recombination with a prokaryotic Gateway™ destination vector **Note:** Also suitable for efficient expression of your gene of interest in other hosts after recombination with a Gateway™ destination vector (*e.g* mammalian, insect, yeast)

High-Throughput Applications

The 480 and 500 reaction pENTR and pENTR/SD Directional TOPO® Cloning Kits are specifically designed to allow production of Gateway™ entry clones for use in high-throughput (HTP) applications. In these kits, the pENTR TOPO® vector is provided in bulk, and chemically competent TOP10 *E. coli* are provided in a choice of two formats:

- Cells are provided in bulk aliquots of 5 ml to allow simple transfer of the cells from a sterile trough into a 96-well plate containing the TOPO® Cloning reaction (Catalog nos. K2400-500 and K2420-500).
- Cells are provided pre-aliquoted in 96-well plates (in 12-well stripwells) to allow addition of the TOPO® Cloning reaction to the cells (Catalog nos. K2400-480 and K2420-480).

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Overview, continued

The Gateway™ Technology

Gateway™ is a universal cloning technology that takes advantage of the site-specific recombination properties of bacteriophage lambda (Landy, 1989) to provide a rapid and highly efficient way to move your gene of interest into multiple vector systems. To express your gene of interest using the Gateway™ Technology, simply:

1. TOPO® Clone your blunt-end PCR product into one of the pENTR TOPO® vectors to generate an entry clone.
2. Generate an expression construct by performing an LR recombination reaction between the entry clone and a Gateway™ destination vector of choice.
3. Introduce your expression construct into the appropriate host (*e.g.* bacterial, mammalian, yeast, insect) and express your recombinant protein.

For more information about the Gateway™ Technology, refer to the Gateway™ Technology manual, see our Web site (www.invitrogen.com) or call Technical Service (see page 32). The Gateway™ Technology manual is available for downloading from our Web site or by contacting Technical Service.

Features of the pENTR TOPO® Vectors

The pENTR/D-TOPO® and pENTR/SD/D-TOPO® vectors are designed to facilitate rapid, directional TOPO® Cloning of blunt-end PCR products for entry into the Gateway™ System. Features of the vectors include:

- *att*L1 and *att*L2 sites for site-specific recombination of the entry clone with a Gateway™ destination vector
- T7 gene 10 translation enhancer and ribosome binding site for efficient translation of the PCR product in prokaryotic systems (pENTR/SD/D-TOPO® only)
- Directional TOPO® Cloning site for rapid and efficient directional cloning of blunt-end PCR products (see page 3 for more information)
- *rrn*B transcription termination sequences to prevent basal expression of the PCR product of interest in *E. coli*
- Kanamycin resistance gene for selection in *E. coli*
- pUC origin for high-copy replication and maintenance of the plasmid in *E. coli*

How Directional TOPO® Cloning Works

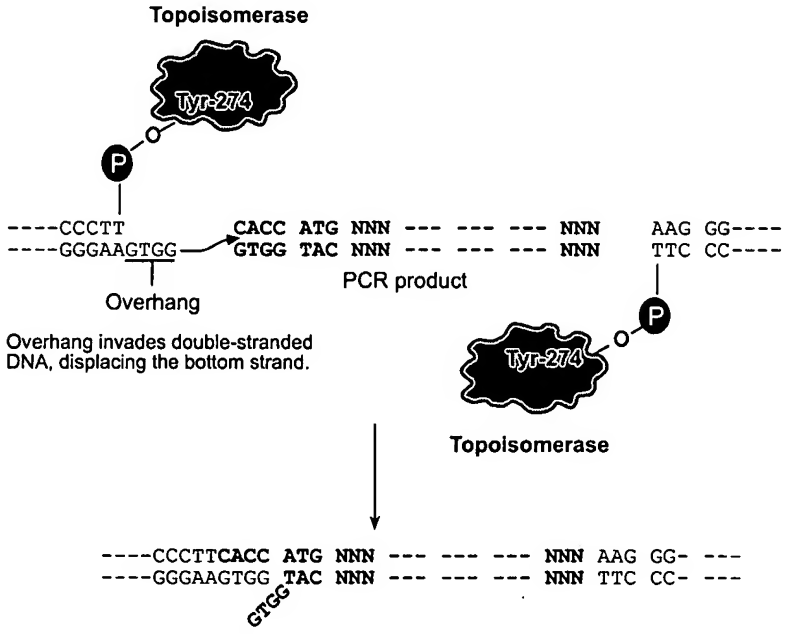
How Topoisomerase I Works

Topoisomerase I from *Vaccinia* virus binds to duplex DNA at specific sites (CCCTT) and cleaves the phosphodiester backbone in one strand (Shuman, 1991). The energy from the broken phosphodiester backbone is conserved by formation of a covalent bond between the 3′ phosphate of the cleaved strand and a tyrosyl residue (Tyr-274) of topoisomerase I. The phospho-tyrosyl bond between the DNA and enzyme can subsequently be attacked by the 5′ hydroxyl of the original cleaved strand, reversing the reaction and releasing topoisomerase (Shuman, 1994). TOPO® Cloning exploits this reaction to efficiently clone PCR products.

Directional TOPO® Cloning

Directional joining of double-strand DNA using TOPO®-charged oligonucleotides occurs by adding a 3′ single-stranded end (overhang) to the incoming DNA (Cheng and Shuman, 2000). This single-stranded overhang is identical to the 5′ end of the TOPO®-charged DNA fragment. At Invitrogen, this idea has been modified by adding a 4 nucleotide overhang sequence to the TOPO®-charged DNA and adapting it to a 'whole vector' format.

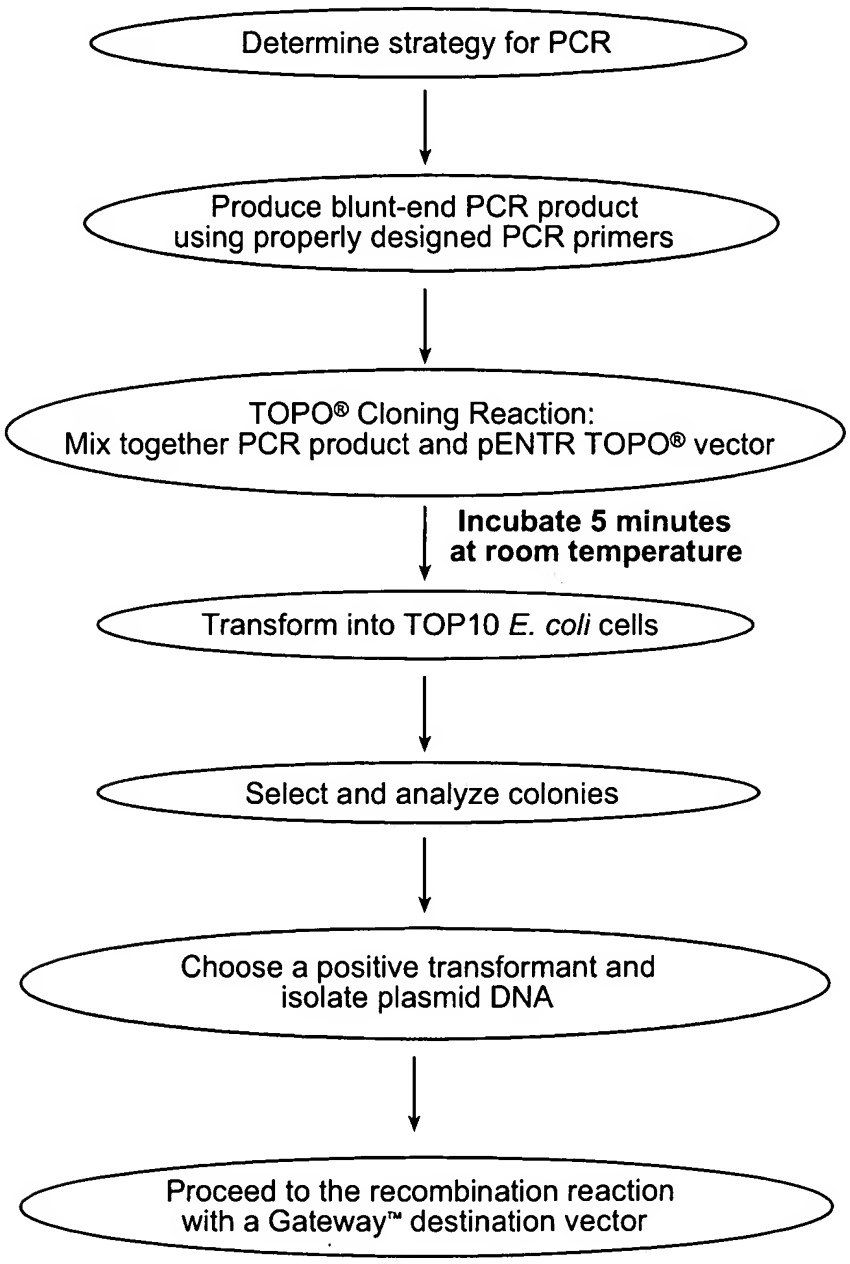
In this system, PCR products are directionally cloned by adding four bases to the forward primer (CACC). The overhang in the cloning vector (GTGG) invades the 5′ end of the PCR product, anneals to the added bases, and stabilizes the PCR product in the correct orientation. Inserts can be cloned in the correct orientation with efficiencies equal to or greater than 90%.



Experimental Outline

Flow Chart

The flow chart below describes the general steps required to produce and clone your blunt-end PCR product.



Methods

Designing PCR Primers

Designing Your PCR Primers

The design of the PCR primers to amplify your gene of interest is critical for expression. Depending on the pENTR TOPO® vector you are using, consider the following when designing your PCR primers.

- Sequences required to facilitate directional cloning
- Sequences required for proper translation initiation of your PCR product
- Whether or not you wish your PCR product to be fused in frame with an N- or C-terminal tag after recombination of your entry clone with a Gateway™ destination vector

Guidelines to Design the Forward PCR Primer

When designing your forward PCR primer, consider the following points below. Refer to page 8 for diagrams of the TOPO® Cloning site for pENTR/D-TOPO® and pENTR/SD/D-TOPO®.

- To enable directional cloning, the forward PCR primer **must** contain the sequence, CACC, at the 5′ end of the primer. The 4 nucleotides, CACC, base pair with the overhang sequence, GTGG, in each pENTR TOPO® vector.
- If you plan to express your PCR product in mammalian cells as a native or C-terminal fusion-tagged protein (following recombination of the entry clone with a Gateway™ destination vector), your sequence of interest should include a Kozak translation initiation sequence with an ATG initiation codon for proper initiation of translation (Kozak, 1987; Kozak, 1991; Kozak, 1990). An example of a Kozak consensus sequence is **(G/A)**NN<u>ATG</u>**G**. Other sequences are possible, but the G or A at position –3 and the G at position +4 are the most critical for function (shown in bold). The ATG initiation codon is underlined.

  Note: If your sequence of interest does not contain an initiation codon within the context of a Kozak sequence, design the forward PCR primer to contain a Kozak sequence at the 5′ end of the primer (see **Example** on the next page).
- If you plan to express your PCR product in mammalian cells as an N-terminal fusion-tagged protein (following recombination of the entry clone with a Gateway™ destination vector), your sequence of interest does not need to contain a Kozak translation initiation sequence. A Kozak sequence is provided by the appropriate destination vector. **Note:** In this case, internal initiation may occur if your PCR product contains an endogenous Kozak sequence.
- If you plan to express your PCR product in **prokaryotic** cells without an N-terminal fusion tag (following recombination of the entry clone with a Gateway™ destination vector), you should TOPO® Clone your PCR product into pENTR/SD/D-TOPO®. pENTR/SD/D-TOPO® contains a T7 gene 10 translational enhancer and a ribosome binding site (RBS) to enable efficient translation of the PCR product in *E. coli.* **To ensure optimal spacing for proper translation, design your forward PCR primer so that the ATG initiation codon of your PCR product directly follows the CACC necessary for directional cloning** (see **Example** on the next page).

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Designing PCR Primers, continued

Example of Forward Primer Design

Below is the DNA sequence of the N-terminus of a theoretical protein and the proposed sequence for your forward PCR primer. The ATG initiation codon is underlined.

DNA sequence: 5'-ATG GGA TCT GAT AAA

Proposed Forward PCR primer: 5'-C ACC ATG GGA TCT GAT AAA

If you design the forward PCR primer as noted above, then:

- The ATG initiation codon falls within the context of a Kozak sequence (see boxed sequence), allowing proper translation initiation of the PCR product in mammalian cells.
- The ATG initiation codon is properly spaced from the RBS (in pENTR/SD/D-TOPO® only), allowing proper translation of the PCR product in prokaryotic cells.

Note

The first three base pairs of the PCR product following the 5' CACC overhang will constitute a functional codon.

Guidelines to Design the Reverse Primer

When designing your reverse PCR primer, consider the following points below. Refer to page 8 for diagrams of the TOPO® Cloning site for pENTR/D-TOPO® and pENTR/SD/D-TOPO®.

- **To ensure that your PCR product clones directionally with high efficiency, the reverse PCR primer MUST NOT be complementary to the overhang sequence GTGG at the 5' end. A one base pair mismatch can reduce the directional cloning efficiency from 90% to 50%, increasing the likelihood of your ORF cloning in the opposite orientation (see Example #1 on the next page).** We have not observed evidence of PCR products cloning in the opposite orientation from a two base pair mismatch.
- If you wish to fuse your PCR product in frame with a C-terminal tag (following recombination of the entry clone with a Gateway™ destination vector), then design the reverse PCR primer to remove the native stop codon in the gene of interest (see **Example #2** on the next page).
- If you **do not** wish to fuse your PCR product in frame with a C-terminal tag (following recombination of the entry clone with a Gateway™ destination vector), then include the native sequence containing the stop codon in the reverse primer or make sure the stop codon is upstream from the reverse PCR primer binding site (see **Example #2** on the next page).

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Designing PCR Primers, continued

Example #1 of Reverse Primer Design

Below is the sequence of the C-terminus of a theoretical protein. You want to fuse the protein in frame with a C-terminal tag (following recombination of the entry clone with a Gateway™ destination vector). The stop codon is underlined.

DNA sequence: AAG TCG GAG CAC TCG ACG ACG GTG TAG-3′

One solution is to design the reverse PCR primer to start with the codon just upstream of the stop codon, but the last two codons contain GTGG (underlined below), which is identical to the 4 bp overhang sequence. As a result, the reverse primer will be complementary to the 4 bp overhang sequence, increasing the probability that the PCR product will clone in the opposite orientation. You want to avoid this situation.

DNA sequence: AAG TCG GAG CAC TCG ACG ACG GTG TAG-3′
Proposed Reverse PCR primer sequence: TG AGC TGC TGC CAC AAA-5′

Another solution is to design the reverse primer so that it hybridizes just downstream of the stop codon, but still includes the C-terminus of the ORF. Note that you will need to replace the stop codon with a codon for an innocuous amino acid such as glycine, alanine, or lysine.

Example #2 of Reverse Primer Design

Below is the sequence for the C-terminus of a theoretical protein. The stop codon is underlined.

...GCG GTT AAG TCG GAG CAC TCG ACG ACT GCA TAG-3′

- To fuse the ORF in frame with a C-terminal tag (supplied by the destination vector after recombination), remove the stop codon by starting with nucleotides homologous to the last codon (TGC) and continue upstream. The reverse primer will be:

  5′-TGC AGT CGT CGA GTG CTC CGA CTT-3′

  This will amplify the C-terminus without the stop codon and allow you to join the ORF in frame with a C-terminal tag.

- If you don't want to join the ORF in frame with a C-terminal tag, simply design the reverse primer to include the stop codon.

  5′-CTA TGC AGT CGT CGA GTG CTC CGA CTT-3′

Important

- Remember that the pENTR TOPO® vectors accept blunt-end PCR products.
- Do not add 5′ phosphates to your primers for PCR. This will prevent ligation into the pENTR TOPO® vectors.
- We recommend that you gel-purify your oligonucleotides, especially if they are long (> 30 nucleotides).

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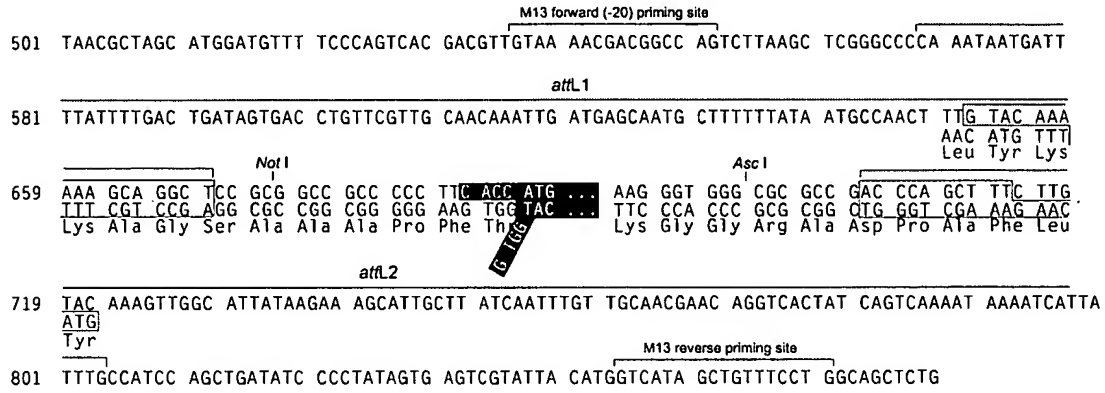
Designing PCR Primers, continued

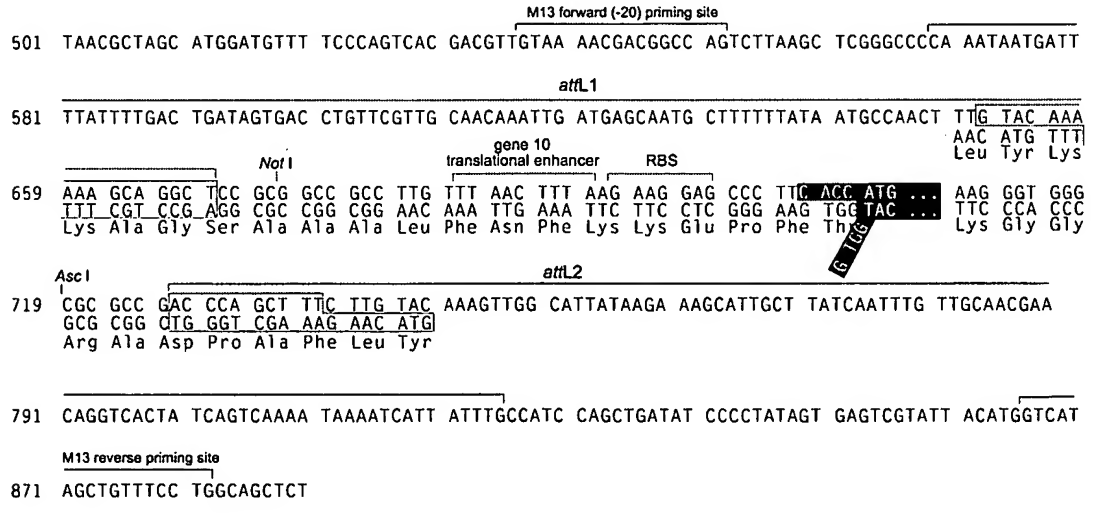
TOPO® Cloning Site for pENTR/D-TOPO®

Use the diagram below to help you design suitable PCR primers to clone your PCR product into pENTR/D-TOPO®. Restriction sites are labeled to indicate the actual cleavage site. The boxed region indicates *att*L sequences in the entry clone that will be transferred into the destination vector following recombination. **The sequence of pENTR/D-TOPO® is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (page 32).** For more information about pENTR/D-TOPO®, see pages 27-28.



TOPO® Cloning Site for pENTR/SD/D-TOPO®

Use the diagram below to help you design suitable PCR primers to clone your PCR product into pENTR/SD/D-TOPO®. Restriction sites are labeled to indicate the actual cleavage site. The boxed region indicates *att*L sequences in the entry clone that will be transferred into the destination vector following recombination. **The sequence of pENTR/SD/D-TOPO® is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (page 32).** For more information about pENTR/SD/D-TOPO®, see pages 29-30.



Producing Blunt-End PCR Products

Introduction

Once you have decided on a PCR strategy and have synthesized the primers, you can produce your blunt-end PCR product. You may use any thermostable, proofreading polymerase including Platinum® *Pfx*, *Pfu*, or Vent® for PCR.

Producing Blunt-End PCR Products

Follow the manufacturer's instructions and recommendations to produce blunt-end PCR products. It is important to optimize PCR conditions to produce a single, discrete PCR product. If you need to gel-purify your fragment, see pages 25-26.

Materials Supplied by the User

You will need the following reagents and equipment for PCR. **Note:** dNTPs (adjusted to pH 8) are provided in the kit.

- Thermocycler and thermostable, proofreading polymerase
- 10X PCR buffer appropriate for your polymerase
- DNA template and primers for PCR product

Producing PCR Products

Set up a 25 µl or 50 µl PCR reaction using the guidelines below:

- Follow the manufacturer's instructions for the DNA polymerase you are using
- Use the cycling parameters suitable for your primers and template
- Use a 7 to 30 minute final extension to ensure that all PCR products are completely extended

After cycling, place the tube on ice or store at –20°C for up to 2 weeks. Proceed to **Checking the PCR Product**, below.

Checking the PCR Product

Remove 5 to 10 µl from each PCR reaction and use agarose gel electrophoresis to verify the quality and quantity of your PCR product. Check for the following:

- Be sure you have a single, discrete band of the correct size. If you do not have a single, discrete band, follow the manufacturer's recommendations for optimizing your PCR with the polymerase of your choice. Alternatively, you may gel-purify the desired product (see pages 25-26).
- Estimate the concentration of your PCR product. We recommend using a **5:1 molar ratio** of PCR product:TOPO® vector to obtain the highest TOPO® Cloning efficiency (*e.g.* use 5-10 ng of a 1 kb PCR product or 10-20 ng of a 2 kb PCR product in a TOPO® Cloning reaction). You may need to adjust the concentration of your PCR product before proceeding to TOPO® Cloning.

Note

If you use Invitrogen's ThermalAce™ polymerase to produce your blunt-end PCR product, note that ThermalAce™ can generate higher yields than other proofreading polymerases. For PCR products in the 0.5-1.0 kb range, we generally dilute the PCR reaction 1:5 in 1X ThermalAce™ buffer before performing the TOPO® Cloning reaction. For PCR products larger than 1.0 kb, dilution may not be required.

Vent® is a registered trademark of New England Biolabs

Setting Up the TOPO® Cloning Reaction

Introduction

Once you have produced the desired PCR product, you are ready to TOPO® Clone it into the pENTR TOPO® vector and transform the recombinant vector into TOP10 *E. coli*. It is important to have everything you need set up and ready to use to ensure that you obtain the best possible results. We suggest that you read the sections entitled **Setting Up the TOPO® Cloning Reaction** (pages 10-11) and **Transforming One Shot® TOP10 Competent Cells** (pages 12-13) before beginning. If this is the first time you have TOPO® Cloned, perform the control reactions on pages 22-23 in parallel with your samples.

If you are TOPO® Cloning in HTP format, you may transform TOP10 *E. coli* using Bulk TOP10 cells (500 reaction kits) or MultiShot™ TOP10 cells (480 reaction kits). Depending on which kit you are using, see the TOPO® Cloning and transformation protocols on pages 15-16 or 17-18.

Salt Solution

Recent experiments at Invitrogen demonstrate that including salt (200 mM NaCl, 10 mM $MgCl_2$) in the TOPO® Cloning reaction may result in an increase in the number of transformants.

From these results, we recommend adding salt to the TOPO® Cloning reaction. A stock salt solution is provided in the kit for this purpose. **Note that the amount of salt added to the TOPO® Cloning reaction varies depending on whether you plan to transform chemically competent cells (provided) or electrocompetent cells (see page x for ordering information).** For this reason two different TOPO® Cloning reactions are provided to help you obtain the best possible results. Read the following information carefully.

Transforming Chemically Competent *E. coli*

For TOPO® Cloning and transformation into chemically competent *E. coli*, adding sodium chloride and magnesium chloride to a final concentration of 200 mM NaCl, 10 mM $MgCl_2$ in the TOPO® Cloning reaction may increase the number of colonies over time. A Salt Solution (1.2 M NaCl, 0.06 M $MgCl_2$) is provided to adjust the TOPO® Cloning reaction to the recommended concentration of NaCl and $MgCl_2$.

Transforming Electrocompetent *E. coli*

For transformation of electrocompetent *E. coli*, the amount of salt in the TOPO® Cloning reaction **must be reduced** to 50 mM NaCl, 2.5 mM $MgCl_2$ to prevent arcing during electroporation. Dilute the Salt Solution 4-fold with water to prepare a 300 mM NaCl, 15 mM $MgCl_2$ solution for convenient addition to the TOPO® Cloning reaction (see the next page).

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Setting Up the TOPO® Cloning Reaction, continued

Setting Up the TOPO® Cloning Reaction

The table below describes how to set up your TOPO® Cloning reaction (6 µl) for eventual transformation into either chemically competent One Shot® TOP10 *E. coli* (provided) or electrocompetent *E. coli*. Additional information on optimizing the TOPO® Cloning reaction for your needs can be found on page 21. If you generated your PCR product using ThermalAce™ polymerase, please note that you may need to dilute your PCR reaction before proceeding (see page 9).

Note: The blue color of the TOPO® vector solution is normal and is used to visualize the solution.

Reagents*	Chemically Competent *E. coli*	Electrocompetent *E. coli*
Fresh PCR product	0.5 to 4 µl	0.5 to 4 µl
Salt Solution	1 µl	--
Dilute Salt Solution (1:4)	--	1 µl
Sterile Water	add to a final volume of 5 µl	add to a final volume of 5 µl
TOPO® vector	1 µl	1 µl

*Store all reagents at –20°C when finished. Salt solution and water can be stored at room temperature or +4°C.

Performing the TOPO® Cloning Reaction

1. Mix reaction gently and incubate for 5 minutes at room temperature (22-23°C).

   Note: For most applications, 5 minutes will yield a sufficient number of colonies for analysis. Depending on your needs, the length of the TOPO® Cloning reaction can be varied from 30 seconds to 30 minutes. For routine subcloning of PCR products, 30 seconds may be sufficient. For large PCR products (> 1 kb) or if you are TOPO® Cloning a pool of PCR products, increasing the reaction time may yield more colonies.

2. Place the reaction on ice and proceed to **Transforming One Shot® TOP10 Competent Cells**, next page. **Note:** You may store the TOPO® Cloning reaction at –20°C overnight.

Transforming One Shot® TOP10 Competent Cells

Introduction

Once you have performed the TOPO® Cloning reaction, you will transform your pENTR TOPO® construct into competent *E. coli*. One Shot® TOP10 Chemically Competent *E. coli* (Box 2) are included with the 20 reaction kit to facilitate transformation, however, you may also transform electrocompetent cells (see page x for ordering information). Protocols to transform chemically competent or electrocompetent *E. coli* are provided in this section.

Materials Supplied by the User

In addition to general microbiological supplies (*i.e.* plates, spreaders), you will need the following reagents and equipment.

- 42°C water bath (or electroporator with cuvettes, optional)
- LB plates containing 50 µg/ml kanamycin (two for each transformation)
- 37°C shaking and non-shaking incubator

Note

There is no blue-white screening for the presence of inserts. Most transformants will contain recombinant plasmids with the PCR product of interest cloned in the correct orientation. Sequencing primers are included in the kit to sequence across an insert in the multiple cloning site to confirm orientation and reading frame.

Preparing for Transformation

For each transformation, you will need one vial of competent cells and two selective plates.

- Equilibrate a water bath to 42°C (for chemical transformation) or set up your electroporator if you are using electrocompetent *E. coli*.
- Warm the vial of SOC medium from Box 2 to room temperature.
- Warm LB plates containing 50 µg/ml kanamycin at 37°C for 30 minutes.
- Thaw <u>on ice</u> 1 vial of One Shot® TOP10 cells from Box 2 for each transformation.

continued on next page

Transforming One Shot® TOP10 Competent Cells, continued

One Shot® TOP10 Chemical Transformation Protocol

1. Add 2 µl of the TOPO® Cloning reaction from **Performing the TOPO® Cloning Reaction**, Step 2, page 11 into a vial of One Shot® TOP10 Chemically Competent *E. coli* and mix gently. **Do not mix by pipetting up and down.**
2. Incubate on ice for 5 to 30 minutes.

   Note: Longer incubations on ice seem to have a minimal effect on transformation efficiency. The length of the incubation is at the user's discretion.
3. Heat-shock the cells for 30 seconds at 42°C without shaking.
4. Immediately transfer the tubes to ice.
5. Add 250 µl of room temperature SOC medium.
6. Cap the tube tightly and shake the tube horizontally (200 rpm) at 37°C for 30 minutes.
7. Spread 50-200 µl from each transformation on a prewarmed selective plate and incubate overnight at 37°C. We recommend that you plate two different volumes to ensure that at least one plate will have well-spaced colonies.
8. An efficient TOPO® Cloning reaction may produce several hundred colonies. Pick ~5 colonies for analysis (see **Analyzing Transformants**, page 19).

Transformation by Electroporation

Use ONLY electrocompetent cells for electroporation to avoid arcing. Do not use the One Shot® TOP10 chemically competent cells for electroporation.

1. Add 2 µl of the TOPO® Cloning reaction from **Performing the TOPO® Cloning Reaction**, Step 2, page 11 into a sterile microcentrifuge tube containing 50 µl of electrocompetent *E. coli* and mix gently. **Do not mix by pipetting up and down. Avoid formation of bubbles.** Transfer the cells to a 0.1 cm cuvette.
2. Electroporate your samples using your own protocol and your electroporator.

   Note: If you have problems with arcing, see the next page.
3. Immediately add 250 µl of room temperature SOC medium.
4. Transfer the solution to a 15 ml snap-cap tube (*i.e.* Falcon) and shake for at least 1 hour at 37°C to allow expression of the kanamycin resistance gene.
5. Spread 20-100 µl from each transformation on a prewarmed selective plate and incubate overnight at 37°C. To ensure even spreading of small volumes, add 20 µl of SOC. We recommend that you plate two different volumes to ensure that at least one plate will have well-spaced colonies.
6. An efficient TOPO® Cloning reaction may produce several hundred colonies. Pick ~5 colonies for analysis (see **Analyzing Transformants**, page 19).

continued on next page

Transforming One Shot® TOP10 Competent Cells, continued

To prevent arcing of your samples during electroporation, the volume of cells should be between 50 and 80 µl (0.1 cm cuvettes) or 100 to 200 µl (0.2 cm cuvettes).

If you experience arcing during transformation, try one of the following suggestions:

- Reduce the voltage normally used to charge your electroporator by 10%
- Reduce the pulse length by reducing the load resistance to 100 ohms
- Ethanol precipitate the TOPO® Cloning reaction and resuspend in water prior to electroporation

HTP TOPO® Cloning and Transformation with Bulk Cells

Description

In this protocol, the TOPO® Cloning reaction is set up in a 96-well U bottom, polystyrene plate (Costar, Catalog no. 3366, 330 µl/well) and the TOP10 competent cells are placed in a trough for dispensing.

Before Starting

- Chill a 96-well metal heating block (VWR, Catalog no. 13259-260) on ice until the block is cold.
- Bring a vial of SOC to room temperature.
- Pre-heat a heat block or thermocycler containing a 96-well metal block to 42°C.

  Note: You may use a water bath, but be careful not to contaminate the cells.
- Thaw 1 tube (5 ml) of TOP10 chemically competent *E. coli* on ice (30-40 minutes).
- Warm LB agar plates containing 50 µg/ml kanamycin to 37°C. If you plan to include a pUC19 control to test the transformation efficiency of the cells, you will need LB agar plates containing 50-100 µg/ml ampicillin.

Controls

For your convenience a 50 µl aliquot of competent cells is provided to perform a test TOPO® Cloning and transformation reaction. In addition, you can include the pUC19 plasmid as an internal control (see **Procedure** below).

Procedure

1. Set up the 6 µl TOPO® Cloning reaction in each well as follows. If you include pUC19 as a control, leave 2-3 wells empty.

PCR product	1 µl
Salt Solution	1 µl
Sterile Water	3 µl
pENTR TOPO® vector	1 µl
Final Volume	6 µl

2. Incubate 5-10 minutes at room temperature.
3. Place the 96-well plate on the cooling block for 5 minutes.
4. If you are including pUC19, add 1 µl (10 pg) of the plasmid to 2-3 empty wells.
5. Pour thawed TOP10 *E. coli* into a sterile trough and immediately dispense 45 µl/well. Gently pipet up and down 1-2 times to mix.
6. Cover the plate with Parafilm® and incubate it on the chilled block for 20 minutes.
7. Transfer the plate to either the pre-warmed heat block or the thermocycler and heat-shock the cells at 42°C for 30 seconds.
8. Transfer the plate back to the cooling block and press down to ensure the plate is in complete contact with the cooling block. Incubate for 1 minute.

continued on next page

HTP TOPO® Cloning and Transformation with Bulk Cells, continued

Procedure, continued

9. Remove the Parafilm® and add 150 µl/well of SOC.
10. Re-cover the plate and incubate the plate at 37°C for 1 hour. **Note:** Gentle shaking (125 rpm) is optional.
11. Plate 50 µl from each well onto LB agar plates containing 50 µg/ml kanamycin. For the pUC19 controls, plate 10 µl of the transformation mixture plus 20 µl of SOC on LB plates containing 100 µg/ml ampicillin. Incubate overnight at 37°C.
12. The next day, select 5-10 colonies and process as desired.

Too Many Colonies

If you obtain too many colonies, reduce the amount of bacterial culture plated and/or dilute the transformation with additional SOC.

HTP TOPO® Cloning and Transformation with MultiShot™ Cells

Description

In this protocol, the TOPO® Cloning reaction is set up in a 96-well plate and 2 µl are transferred to each well of a 96-well MultiShot™ plate containing 15 µl of chemically competent TOP10 *E. coli* per well.

Before Starting

- Chill two 96-well metal heating blocks (VWR, Catalog no. 13259-260) on ice until the blocks are cold.
- Bring a vial of SOC to room temperature.
- Warm LB agar plates containing 50 µg/ml kanamycin to 37°C. If you plan to include a pUC19 control to test the transformation efficiency of the cells, you will need LB agar plates containing 50-100 µg/ml ampicillin.
- Pre-heat a heat block or thermocycler containing a 96-well metal block to 42°C.

  Note: You may use a water bath, but be careful not to contaminate the cells.
- If you are using a thermocycler, program the machine to hold the temperature at 42°C.

Controls

A test plate containing 1 row (12 wells) of TOP10 cells is included to perform test TOPO® Cloning reactions and transformations. In addition, you can include the pUC19 plasmid as an internal control (see **Procedure** below).

Procedure

1. In a 96-well plate, set up the following 6 µl TOPO® Cloning reaction in each well.

PCR product	1 µl
Salt Solution	1 µl
Sterile Water	3 µl
pENTR TOPO® vector	1 µl
Final Volume	6 µl

2. Incubate 5-10 minutes at room temperature.
3. Place the 96-well plate on one of the cooling blocks for 5 minutes.
4. Remove a 96-well MultiShot™ plate of chemically competent TOP10 *E. coli* from the freezer and place it in the second cooling block. Cells should thaw within 30 seconds.
5. Carefully remove the aluminum foil seal.
6. Use a multi-channel pipet to add 2 µl of each TOPO® Cloning reaction (~3.3 ng) to each well of the 96-well plate containing cells. Keep the volume around 2 µl for uniform results. For the pUC19 control, add 1 µl (10 pg) of the DNA.

continued on next page

HTP TOPO® Cloning and Transformation with MultiShot™ Cells, continued

Procedure, continued

7. Cover the cells with the supplied plastic lid and incubate the cells and DNA in the chilled block for 20 minutes.
8. Transfer the cell plate to either the pre-warmed heat block or thermocycler and heat-shock for 30 seconds at 42°C.
9. Transfer the cell plate back to a cooling block, press the plate into the block, and allow the plate to cool for 1 minute.
10. Remove the plastic lid and add 90 µl SOC to each well.
11. Cover the plate with the lid and incubate the plate at 37°C for 1 hour. **Note:** Gentle shaking (125 rpm) is optional.
12. Plate 100 µl from each well onto LB agar plates containing 50 µg/ml kanamycin. For the pUC19 controls, plate 10 µl of the transformation mixture plus 20 µl of SOC on LB plates containing 100 µg/ml ampicillin. Incubate overnight at 37°C.

Too Many Colonies

If you obtain too many colonies, you can reduce the amount of cells plated or dilute the TOPO® Cloning reactions with sterile water or TE buffer prior to adding the reaction to the cells.

Analyzing Transformants

Analyzing Positive Clones

1. Pick 5 colonies and culture them overnight in LB or SOB medium containing 50-100 µg/ml kanamycin.
2. Isolate plasmid DNA using your method of choice. If you need ultra-pure plasmid DNA for automated or manual sequencing, we recommend using the S.N.A.P.™ MidiPrep Kit (Catalog no. K1910-01).
3. Analyze the plasmids by restriction analysis to confirm the presence and correct orientation of the insert. Use a restriction enzyme or a combination of enzymes that cut once in the vector and once in the insert.

Sequencing

You may sequence your construct to confirm that your gene is cloned in the correct orientation. The M13 Forward (-20) and M13 Reverse primers are included in the kit to help you sequence your insert (see the diagrams on page 8 for the location of the priming sites). The M13 Forward (-20) and M13 Reverse primers are available separately from Invitrogen (see page x for ordering information).

Important

If you download the sequence for pENTR/D-TOPO® or pENTR/SD/D-TOPO® from our Web site, note that the overhang sequence (GTGG) will be shown already hybridized to CACC. No DNA sequence analysis program allows us to show the overhang without the complementary sequence.

Analyzing Transformants by PCR

You may analyze positive transformants using PCR. For PCR primers, use a combination of the M13 Forward (-20) primer or the M13 Reverse primer and a primer that hybridizes within your insert. You will have to determine the amplification conditions. If you are using this technique for the first time, we recommend performing restriction analysis in parallel. Artifacts may be obtained because of mispriming or contaminating template. The protocol below is provided for your convenience. Other protocols are suitable.

Materials Needed:

PCR Super Mix High Fidelity (Invitrogen, Catalog no. 10790-020)

Appropriate forward and reverse PCR primers (20 µM each)

Protocol:

1. For each sample, aliquot 48 µl of PCR SuperMix High Fidelity into a 0.5 ml microcentrifuge tube. Add 1 µl each of the forward and reverse PCR primer.
2. Pick 5 colonies and resuspend them individually in 50 µl of the PCR SuperMix containing PCR primers (remember to make a patch plate to preserve the colonies for further analysis).
3. Incubate reaction for 10 minutes at 94°C to lyse cells and inactivate nucleases.
4. Amplify for 20 to 30 cycles.
5. For the final extension, incubate at 72°C for 10 minutes. Store at +4°C.
6. Visualize by agarose gel electrophoresis.

continued on next page

Analyzing Transformants, continued

Important

If you have problems obtaining transformants or the correct insert, perform the control reactions described on page 22-23. These reactions will help you trouble-shoot your experiment.

Long-Term Storage

Once you have identified the correct clone, be sure to purify the colony and make a glycerol stock for long-term storage. We recommend that you store a stock of plasmid DNA at -20°C.

1. Streak the original colony out for single colony on LB plates containing 50 µg/ml kanamycin.
2. Isolate a single colony and inoculate into 1-2 ml of LB containing 50 µg/ml kanamycin.
3. Grow until culture reaches stationary phase.
4. Mix 0.85 ml of culture with 0.15 ml of sterile glycerol and transfer to a cryovial.
5. Store at -80°C.

Recombining the Entry Construct with a Destination Vector

Once you have obtained your entry clone, you may recombine the pENTR TOPO® construct with any Gateway™ destination vector of choice to generate an expression clone. This LR recombination reaction is mediated by LR Clonase™ Enzyme Mix, a cocktail of recombination proteins. LR Clonase™ Enzyme Mix is available from Invitrogen (see page x for ordering information).

For more information about the LR recombination reaction and the LR Clonase™ Enzyme Mix, refer to the Gateway™ Technology manual. To obtain more information about the Gateway™ destination vectors available, see our Web site (www.invitrogen.com) or call Technical Service (see page 32).

Optimizing the TOPO® Cloning Reaction

Introduction

The information below will help you optimize the TOPO® Cloning Reaction for your particular needs.

Faster Subcloning

The high efficiency of TOPO® Cloning allows you to streamline the cloning process. If you routinely clone PCR products and wish to speed up the process, consider the following:

- Incubate the TOPO® Cloning reaction for only 30 seconds instead of 5 minutes.

  You may not obtain the highest number of colonies, but with the high efficiency of TOPO® Cloning, most of the transformants will contain your insert.
- After adding 3 µl of the TOPO® Cloning reaction to chemically competent cells, incubate on ice for only 5 minutes.

  Increasing the incubation time to 30 minutes does not significantly improve transformation efficiency.

More Transformants

If you are TOPO® Cloning large PCR products, toxic genes, or cloning a pool of PCR products, you may need more transformants to obtain the clones you want. To increase the number of colonies:

- Incubate the salt-supplemented TOPO® Cloning reaction for 20 to 30 minutes instead of 5 minutes.

  Increasing the incubation time of the salt-supplemented TOPO® Cloning reaction allows more molecules to ligate, increasing the transformation efficiency. Addition of salt appears to prevent topoisomerase I from rebinding and nicking the DNA after it has ligated the PCR product and dissociated from the DNA.
- Titrate the amount of PCR product used in the TOPO® Cloning reaction for maximum colony output.

Cloning Dilute PCR Products

To clone dilute PCR products, you may:

- Increase the amount of the PCR product
- Incubate the TOPO® Cloning reaction for 20 to 30 minutes
- Concentrate the PCR product

Appendix

Performing the Control Reactions

Introduction

We recommend performing the following control TOPO® Cloning reactions the first time you use the 20 reaction kit to help you evaluate your results. Performing the control reactions involves producing a control PCR product using the reagents included in the kit and using this product directly in a TOPO® Cloning reaction.

Before Starting

For each transformation, prepare two LB plates containing 50 µg/ml kanamycin.

Producing the Control PCR Product

Use your thermostable, proofreading polymerase and the appropriate buffer to amplify the control PCR product. Follow the manufacturer's recommendations for the polymerase you are using.

1. To produce the 750 bp control PCR product, set up the following 50 µl PCR:

Control DNA Template (100 ng)	1 µl
10X PCR Buffer (appropriate for enzyme)	5 µl
dNTP Mix	0.5 µl
Control PCR Primers (0.1 µg/µl each)	1 µl
Sterile Water	41.5 µl
Thermostable polymerase (1-2.5 units/µl)	1 µl
Total Volume	50 µl

2. Overlay with 70 µl (1 drop) of mineral oil.
3. Amplify using the following cycling parameters:

Step	Time	Temperature	Cycles
Initial Denaturation	2 minutes	94°C	1X
Denaturation	1 minute	94°C	25X
Annealing	1 minute	55°C	
Extension	1 minute	72°C	
Final Extension	7 minutes	72°C	1X

4. Remove 10 µl from the reaction and analyze by agarose gel electrophoresis. A discrete 750 bp band should be visible. Proceed to the **Control TOPO® Cloning Reactions**, next page.

continued on next page

Performing the Control Reactions, continued

Control TOPO® Cloning Reactions

Using the control PCR product produced on the previous page and the pENTR TOPO® vector, set up two 6 µl TOPO® Cloning reactions as described below. If you plan to transform electrocompetent *E. coli*, use Dilute Salt Solution in place of the Salt Solution.

1. Set up control TOPO® Cloning reactions:

Reagent	"Vector Only"	"Vector + PCR Insert"
Sterile Water	4 µl	3 µl
Salt Solution	1 µl	1 µl
Control PCR Product	--	1 µl
pENTR TOPO® vector	1 µl	1 µl

2. Incubate at room temperature for **5 minutes** and place on ice.

   Transform 2 µl of each reaction into separate vials of One Shot® TOP10 cells (page 13).

3. Spread 50-200 µl of each transformation mix onto LB plates containing 50 µg/ml kanamycin. Be sure to plate two different volumes to ensure that at least one plate has well-spaced colonies.
4. Incubate overnight at 37°C.

Analysis of Results

Hundreds of colonies from the vector + PCR insert reaction should be produced. To analyze the transformations, isolate plasmid DNA and digest with the appropriate restriction enzyme as listed below. The table below lists the digestion patterns that you should see for inserts that are cloned in the correct orientation or in the reverse orientation.

Vector	Restriction Enzyme	Expected Digestion Patterns (bp)
pENTR/D-TOPO®	*Not* I	Correct orientation: 127, 3203 Reverse orientation: 646, 2684 Empty vector: 2580
pENTR/SD/D-TOPO®	*Not* I	Correct orientation: 148, 3203 Reverse orientation: 667, 2684 Empty vector: 2601

Greater than 90% of the colonies should contain the 750 bp insert in the correct orientation.

Relatively few colonies should be produced in the vector-only reaction.

continued on next page

Performing the Control Reactions, continued

Transformation Control

pUC19 plasmid is included to check the transformation efficiency of the One Shot® TOP10 competent cells. Transform one vial of One Shot® TOP10 cells with 10 pg of pUC19 using the protocol on page 13. Plate 10 µl of the transformation mixture plus 20 µl of SOC on LB plates containing 100 µg/ml ampicillin. Transformation efficiency should be ~1 x 10^9 cfu/µg DNA.

Factors Affecting Cloning Efficiency

Please note that lower cloning efficiencies will result from the following variables. Most of these are easily corrected, but if you are cloning large inserts, you may not obtain the expected 90% directional cloning efficiency.

Variable	Solution
Low efficiency of directional cloning	• Forward primer should contain CACC at the 5′ end. • Reverse primer is complementary to the overhang at the 5′ end. Re-design primer to avoid base pairing to the overhang.
pH>9 in PCR amplification reaction	Check the pH of the PCR amplification reaction and adjust with 1 M Tris-HCl, pH 8.
Incomplete extension during PCR	Be sure to include a final extension step of 7 to 30 minutes during PCR. Longer PCR products will need a longer extension time.
Cloning large inserts (>1 kb)	• Increase amount of insert or gel-purify as described on pages 25-26. • Increase the incubation time of the TOPO® Cloning reaction.
Excess (or overly dilute) PCR product	Reduce (or concentrate) the amount of PCR product.
PCR cloning artifacts ("false positives")	TOPO® Cloning is very efficient for small fragments (< 100 bp) present in certain PCR reactions. Gel-purify your PCR product (pages 25-26) or optimize your PCR.

Gel Purifying PCR Products

Introduction

Smearing, multiple banding, primer-dimer artifacts, or large PCR products (>3 kb) may necessitate gel purification. If you wish to purify your PCR product, be extremely careful to remove all sources of nuclease contamination. There are many protocols to isolate DNA fragments or remove oligonucleotides. Please refer to *Current Protocols in Molecular Biology*, Unit 2.6 (Ausubel *et al.*, 1994) for the most common protocols. Three simple protocols are provided below.

Note

The cloning efficiency may decrease with purification of the PCR product (*e.g.* PCR product too dilute). You may wish to optimize your PCR to produce a single band (see **Producing Blunt-End PCR Products**, page 9).

Using the S.N.A.P.™ Gel Purification Kit

The S.N.A.P.™ Gel Purification Kit available from Invitrogen (Catalog no. K1999-25) allows you to rapidly purify PCR products from regular agarose gels.

1. Electrophorese amplification reaction on a 1 to 5% regular TAE agarose gel. **Note**: Do not use TBE to prepare agarose gels. Borate interferes with the sodium iodide step, below.
2. Cut out the gel slice containing the PCR product and melt it at 65°C in 2 volumes of the 6 M sodium iodide solution.
3. Add 1.5 volumes Binding Buffer.
4. Load solution (no more than 1 ml at a time) from Step 3 onto a S.N.A.P.™ column. Centrifuge 1 minute at 3000 x g in a microcentrifuge and discard the supernatant.
5. If you have solution remaining from Step 3, repeat Step 4.
6. Add 900 µl of the Final Wash Buffer.
7. Centrifuge 1 minute at full speed in a microcentrifuge and discard the flow-through.
8. Repeat Step 7.
9. Elute the purified PCR product in 40 µl of TE or sterile water. Use 4 µl for the TOPO® Cloning reaction and proceed as described on page 11.

Quick S.N.A.P.™ Method

An even easier method is to simply cut out the gel slice containing your PCR product, place it on top of the S.N.A.P.™ column bed, and centrifuge at full speed for 10 seconds. Use 1-2 µl of the flow-through in the TOPO® Cloning reaction (page 11) Be sure to make the gel slice as small as possible for best results.

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Gel Purifying PCR Products, continued

Low-Melt Agarose Method

If you prefer to use low-melt agarose, use the procedure below. Note that gel purification will result in a dilution of your PCR product and a potential loss of cloning efficiency.

1. Electrophorese as much as possible of your PCR reaction on a low-melt agarose gel (0.8 to 1.2%) in TAE buffer.
2. Visualize the band of interest and excise the band.
3. Place the gel slice in a microcentrifuge tube and incubate the tube at 65°C until the gel slice melts.
4. Place the tube at 37°C to keep the agarose melted.
5. Add 4 µl of the melted agarose containing your PCR product to the TOPO® Cloning reaction as described on page 11.
6. Incubate the TOPO® Cloning reaction **at 37°C for 5 to 10 minutes**. This is to keep the agarose melted.
7. Transform 2 to 4 µl directly into One Shot® TOP10 cells using the method on page 13.

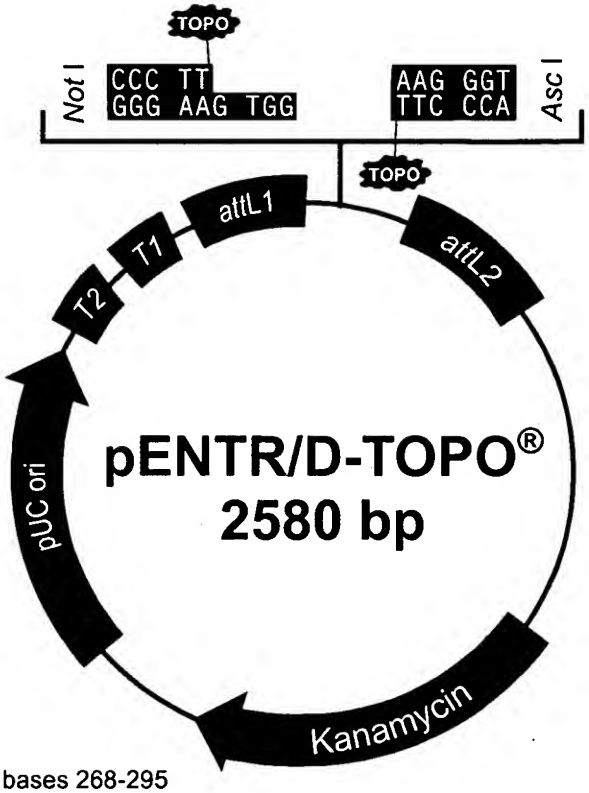
Note

The cloning efficiency may decrease with purification of the PCR product. You may wish to optimize your PCR to produce a single band.

Map and Features of pENTR/D-TOPO

pENTR/D-TOPO® Map

The figure below shows the features of pENTR/D-TOPO® vector. **The complete sequence of pENTR/D-TOPO® is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 32).**



Comments for pENTR/D-TOPO®
2580 nucleotides

*rrn*B T2 transcription termination sequence: bases 268-295
*rrn*B T1 transcription termination sequence: bases 427-470
M13 forward (-20) priming site: bases 537-552
*att*L1: bases 569-668 (complementary strand)
TOPO® recognition site 1: bases 680-684
Overhang: bases 685-688
TOPO® recognition site 2: bases 689-693
*att*L2: bases 705-804
M13 reverse priming site: bases 845-861
Kanamycin resistance gene: bases 974-1783
pUC origin: bases 1904-2577

continued on next page

Map and Features of pENTR/D-TOPO®, continued

Features of pENTR/D-TOPO®

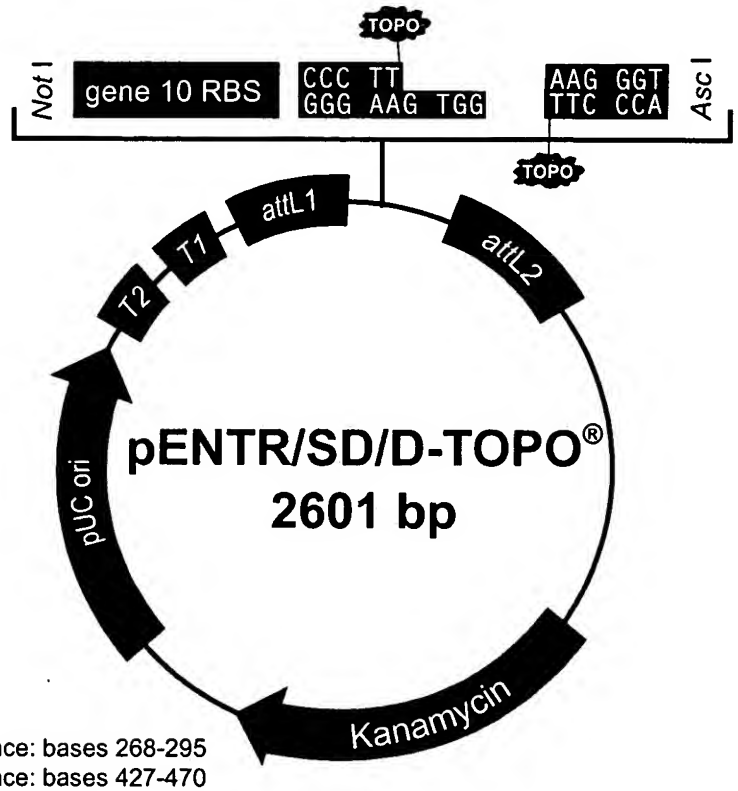
pENTR/D-TOPO® (2580 bp) contains the following elements. All features have been functionally tested.

Feature	Benefit
*rrn*B T1 and T2 transcription termination sequences	Reduces potential toxicity in *E. coli* by preventing basal expression of the PCR product.
M13 forward (-20) priming site	Permits sequencing of the insert.
*att*L1 and *att*L2 sites	Bacteriophage λ-derived recombination sequences that allow recombinational cloning of a gene of interest in the entry construct with a Gateway™ destination vector (Landy, 1989).
TOPO® Cloning site (directional)	Permits rapid, directional cloning of your PCR product.
M13 reverse priming site	Allows sequencing of the insert.
Kanamycin resistance gene	Allows selection of the plasmid in *E. coli*.
pUC origin of replication (*ori*)	Permits high-copy replication and maintenance in *E. coli*.

Map and Features of pENTR/SD/D-TOPO®

pENTR/SD/D-TOPO® Map

The figure below shows the features of pENTR/SD/D-TOPO® vector. **The complete sequence of pENTR/SD/D-TOPO® is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 32).**



Comments for pENTR/SD/D-TOPO®
2601 nucleotides

*rrn*B T2 transcription termination sequence: bases 268-295
*rrn*B T1 transcription termination sequence: bases 427-470
M13 forward (-20) priming site: bases 537-552
attL1: bases 569-668 (complementary strand)
T7 gene 10 translational enhancer: bases 684-692
Ribosome binding site: bases 694-700
TOPO® recognition site 1: bases 701-705
Overhang: bases 706-709
TOPO® recognition site 2: bases 710-714
attL2: bases 726-825
M13 reverse priming site: bases 866-882
Kanamycin resistance gene: bases 995-1804
pUC origin: bases 1925-2598

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Map and Features of pENTR/SD/D-TOPO®, continued

Features of pENTR/SD/D-TOPO®

pENTR/SD/D-TOPO® (2601 bp) contains the following elements. All features have been functionally tested.

Feature	Benefit
*rrn*B T1 and T2 transcription termination sequences	Reduces potential toxicity in *E. coli* by preventing basal expression of the PCR product (Orosz *et al.*, 1991).
M13 forward (-20) priming site	Permits sequencing of the insert.
T7 gene 10 translational enhancer	Sequence from bacteriophage T7 gene 10 that optimizes translation initiation (Olins *et al.*, 1988).
Ribosome binding site (RBS)	Optimally spaced from the TOPO® Cloning site for efficient translation of the PCR product.
*att*L1 and *att*L2 sites	Bacteriophage λ-derived recombination sequences that allow recombinational cloning of a gene of interest in the entry construct with a Gateway™ destination vector (Landy, 1989).
TOPO® Cloning site (directional)	Permits rapid, directional cloning of your PCR product.
M13 reverse priming site	Allows sequencing of the insert.
Kanamycin resistance gene	Allows selection of the plasmid in *E. coli*.
pUC origin of replication (*ori*)	Permits high-copy replication and maintenance in *E. coli*.

Recipes

LB (Luria-Bertani) Medium and Plates

Composition:

1.0% Tryptone
0.5% Yeast Extract
1.0% NaCl
pH 7.0

1. For 1 liter, dissolve 10 g tryptone, 5 g yeast extract, and 10 g NaCl in 950 ml deionized water.
2. Adjust the pH of the solution to 7.0 with NaOH and bring the volume up to 1 liter.
3. Autoclave on liquid cycle for 20 minutes. Allow solution to cool to ~55°C and add antibiotic, if needed.
4. Store at room temperature or at +4°C.

LB agar plates

1. Prepare LB medium as above, but add 15 g/L agar before autoclaving.
2. Autoclave on liquid cycle for 20 minutes.
3. After autoclaving, cool to ~55°C, add antibiotic and pour into 10 cm plates.
4. Let harden, then invert and store at +4°C, in the dark.

Technical Service

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2. Follow instructions on the page and fill out all the required fields.
3. To request additional MSDSs, click the 'Add Another' button.
4. All requests will be faxed unless another method is selected.
5. When you are finished entering information, click the 'Submit' button. Your MSDS will be sent within 24 hours.

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Technical Service, continued

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Product Qualification

Introduction

This section describes the criteria used to qualify the components of the pENTR and pENTR/SD Directional TOPO® Cloning Kits.

Vectors

Restriction analysis with the enzymes listed below is performed on each lot of pENTR, the parent vector of pENTR/D-TOPO® and pENTR/SD/D-TOPO®. The supercoiled vector is qualified by restriction digest prior to adaptation with topoisomerase I. Restriction digests must demonstrate the correct banding pattern when electrophoresed on an agarose gel (see below). Note that the restriction sites used to qualify the parent vector may no longer be present in the topoisomerase I-adapted vectors. The parental vector, pENTR, is 2577 bp in size.

Vector	Restriction Enzyme	Expected Fragments (bp)
pENTR	*Afl* III	236, 696, 1645
	Not I	2577
	Nsi I	266, 2311

TOPO® Cloning Efficiency

The pENTR/D-TOPO® and pENTR/SD/D-TOPO® vectors are lot-qualified using the control reagents included in the kit. Under conditions described on pages 22-23, a 750 bp control PCR product is amplified using a forward primer containing CACC at its 5' end and a reverse primer. The PCR product is TOPO® Cloned into the pENTR/D-TOPO® or pENTR/SD/D-TOPO® vector and transformed into the One Shot® TOP10 chemically competent *E. coli* included with the kit.

Each lot of vector should yield greater than 85% cloning efficiency. Forty transformants are characterized using directional PCR. Of the transformants characterized, greater than 90% should contain an insert in the correct orientation.

Primers

Primers are lot-qualified by DNA sequencing experiments using the dideoxy chain termination technique.

TOP10 Chemically Competent *E. coli*

All competent cells are tested for transformation efficiency using the control plasmid included in the TOP10 Chemically Competent Cell kit. Transformed cultures are plated on LB plates containing 100 µg/ml ampicillin and the transformation efficiency is calculated. Test transformations are performed in duplicate. Transformation efficiency should be:

- Greater than 1 x 10^9 cfu/µg plasmid DNA for the One Shot® TOP10 *E. coli* (supplied in the 20 reaction kit)
- Greater than 1 x 10^8 cfu/µg plasmid DNA for the MultiShot™ or Bulk TOP10 *E. coli* (supplied in the 480 and 500 reaction kits, respectively)

In addition, untransformed cells are tested for the appropriate antibiotic sensitivity and lack of phage contamination.

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